Low Concentrations of the Isoflavone Genistein Influence in vitro Asexual Reproduction and Growth of *Phytophthora sojae*

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ABSTRACT

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Isolates of *Phytophthora sojae* were evaluated for their sensitivity to a series of low concentrations of genistein (0.01, 0.1, 1, 3, and 10 µg/ml) during various stages of growth and asexual reproduction. Genistein was applied during mycelial growth, formation of zoosporangia, release of zoospores, and zoospore germination. At 10 µg/ml, genistein inhibited radial growth of all isolates in solid culture. Changes in fungal morphology, such as increased branching and swelling of hyphae, were observed at all concentrations of genistein tested. Growth of mycelium in broth containing 10 µg of genistein per ml decreased colonies resulting from subsequently formed and germinated zoospores, even when genistein was removed during zoosporangia formation. Genistein also decreased zoosporangia production and release of zoospores. The strongest inhibition of *P. sojae* reproduction was obtained when genistein was applied during zoosporangia formation; this occurred at concentrations as low as 0.01 to 1 µg/ml. Genistein in the medium containing zoospores caused a general reduction in asexual reproduction across isolates, with 10 µg/ml causing the most dramatic reduction. There was strong evidence of intraspecific variation in the responses of the isolates of *P. sojae* in all processes tested.

Phytophthora sojae M.J. Kaufmann & J.W. Gerdemann (synonym *P. megasperma* Drechs. f. sp. *glycinea* T. Kuan & D.C. Erwin) is one of the most economically important pathogens of soybean (*Glycine max* (L.) Merr.), causing severe root and stem rot. As with other *Phytophthora* species, the main infective units are motile zoospores that are attracted to the roots. Infection occurs after aggregation and encystment of zoospores in the zone of root elongation.

P. sojae is a host-specific pathogen, and its specialization implies a mechanism for recognition that most likely requires an exchange of signals between the host and pathogen. Genistein, as well as daidzein and their conjugates, is a secondary isoflavone metabolite in soybean roots, hypocotyls, and cotyledons (10). Some isoflavones are thought to act as signal molecules for symbionts and to participate in transduction pathways leading to physiological and developmental changes in plant pathogens (5,17). Isoflavones have previously increased the initiation of mycorrhizae (14), and it is well documented that genistein and daidzein activate the nodulation genes of the soybean-specific *Bradyrhizobium japonicum* (1,4) analogously to the induction of nod genes by flavones in Rhizobium-legume symbioses (12).

The significance of isoflavones for fungal pathogens such as *P. sojae* has yet to be investigated. It is well known that isoflavones stimulate rapid encystment of zoospores in parasitic Oomycetes (2,5,13). Also, taxis of zoospores of *P. sojae* to soybean isoflavones at extremely low concentrations, to which other *Phytophthora* spp. did not respond, was demonstrated in vitro (13). Morris and Ward (13) suggested *P. sojae* has developed mechanisms for recognition of the same chemical signals as *B. japonicum*, although not for the purpose of symbiosis. To our knowledge, there have been no studies conducted on the effect of genistein on the reproduction of *P. sojae*. Graham (10) showed rapid release of genistein from soybean seeds and roots in vitro and suggested that at very low concentrations (less than 10 µM) in soil, this isoflavone may act as a signal molecule for reproduction and taxis.

The objectives of the present study were to investigate whether low concentrations of genistein can affect the asexual reproduction of *P. sojae* in vitro and to determine whether there is intraspecific variability in the response of *P. sojae* to genistein.

MATERIALS AND METHODS

Selected isolates of *P. sojae* were evaluated for their sensitivity to a series of concentrations of genistein during growth and asexual reproduction. Isolates were derived in part from the cultures MSU 01 (a mixture of races 2 and 11), MSU 02 (race 3), and MSU 03 (race 25), all isolated from Michigan soybean plants in 1993 by R. Kaitany of Michigan State University, East Lansing (race identification was done by A. F. Schmitthenner, Ohio Agricultural Research and Development Center, Wooster). The isolates MSU 01-976, MSU 01-P, MSU 02-1365, and MSU 03-05 are single-zoospore cultures from the corresponding field isolates; MSU 02-1365-R and MSU 03-05-R were obtained from genistein-resistant (10 µg/ml) zoospores of the corresponding field and single-zoospore isolates. Isolates R4 (race 4, B63R4.38), R25 (race 25, B208R25.81), and WR4 (avirulent strain of race 4, WR4.1.3.22) were provided by A. F. Schmitthenner. All isolates were maintained on V8-juice agar (200 ml of clarified V8 juice, 2 g of $CaCO_3$ per liter, 1.5% Bacto agar) at 15°C in the dark. Genistein (4′,5, 7-trihydroxyisoflavone) was synthesized by and obtained from M. Nair (Michigan State University). The concentrations of

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genistein tested were 0, 0.01, 0.1, 1, 3, and 10 µg/ml (0, 0.037, 0.37, 3.7, 11.1, and 37 µM, respectively).

The processes examined in our study include mycelial growth and the intermediary stages of asexual sporulation, including removal of the growth medium and its substitution by water (the key step in zoosporangia production), cold shock resulting in the release of zoospores, and zoospore germination on a rich solid medium. The formation of colonies is used as an indication of zoospore germination.

Influence of genistein on radial growth of *P. sojae* in solid culture. Isolates were plated on V8-juice agar containing either 0, 3, or 10 µg of genistein per ml of medium. Colonies were grown for 5 days, after which the radial growth of the mycelium was measured. Absolute data for controls are given, and data for genistein concentrations are presented as the percentage of control for standardization.

Induction of sporangium formation. Three mycelial plugs (each 6 mm in diameter) from the edge of an actively growing colony (10 days old) of each isolate were transferred to a sterile petri plate, fragmented thoroughly, and flooded with 7 ml of 50% V8-juice broth. After 48 h of incubation at 19°C, the broth was removed, and plates were washed once with sterile deionized distilled water and then with the mineral salts solution of Chen and Zentmyer (3). Resultant mycelial preparations were flooded with 7 ml of sterile deionized distilled water containing various concentrations of genistein and incubated under fluorescent light at 18°C. After 5 to 12 h, zoosporangia were formed and were ready to release zoospores.

Influence of genistein on zoospore release and zoosporangium germination. After genistein-induced zoosporangium formation, the cultures were incubated at 5°C for 30 min. When the cultures were returned to room temperature (23°C) zoospores were released. One hundred microliters of each zoospore suspension was transferred into micropreparation wells, and 2 drops of a 0.1% trypan blue solution in lactoglycerol were added to each well. The number of zoospores in each sample was determined by counting under a light microscope at 250×. After zoospores were released, the remaining populations of zoosporangia, which did not release zoospores, were assessed for direct germination by light microscopy.

Influence of genistein on zoospore germination. Aliquots (100 µl) of zoospore suspensions, obtained in the absence of genistein, were plated on V8-juice agar containing different concentrations of genistein. It was not possible to standardize the zoospore concentrations of the various isolates because the time required for these manipulations altered the ratio of motile and encysted zoospores and their germination. This phenomenon also was observed for *Pythium aphanidermatum* (7). Therefore, the treatments for each isolate were compared with untreated controls of the same isolate. After zoospores were incubated on genistein-amended medium for 3 days, the number of fungal colonies formed were counted. These values are expressed as the percentage of colonies formed by the untreated control.

In a second experiment, isolates of *P. sojae* were cultured as previously described on V8-juice agar amended with genistein at various concentrations during growth and zoospore induction. The resulting zoospore suspensions with defined titers were plated on V8-juice agar without genistein. The resulting colonies were counted. This number was expressed as a percentage of the colonies formed by the untreated control.

Influence of genistein during mycelial growth and reproduction. Six isolates were exposed to different concentrations of genistein only during growth and only during growth and zoospore production. To determine whether mycelial growth in genistein can influence asexual reproduction, two concentrations that previously affected the growth of *P. sojae* on V8 agar were used in four treatments: (i) mycelial growth in the presence of genistein at 3 µg/ml, then removal of genistein by washing; (ii) mycelial growth and subsequent zoosporangium and zoospore production in the presence of genistein at 3 µg/ml; (iii) mycelial growth in the presence of genistein at 10 µg/ml, then removal of genistein by washing; and (iv) mycelial growth and subsequent zoosporangium and zoospore production in the presence of genistein at 10 µg/ml. The number of resulting colonies was expressed as a percentage of the colonies formed without exposure to genistein.

Experimental design and data analysis. All experiments included at least three replicates per treatment and were performed at least three times, with similar results. Unless otherwise stated, only statistically significant results are discussed. Differences among isolates, within a given concentration, were separated by Tukey's significant difference test. Effects of increasing genistein concentrations within isolates were assessed by regression analysis. F and probability (P) values are given in the figure legends. Tabular and graphic data describe the results from one representative experiment.

RESULTS

Influence of genistein on radial growth in solid culture. Growth of *P. sojae* in solid media was influenced by the presence of genistein at 3 and 10 µg/ml (Table 1). Concentrations lower than these did not significantly affect the size of the colonies (data not shown). For several isolates, genistein concentrations of 3 µg/ml caused marked reductions of growth relative to the control. At 10 µg/ml, growth of all isolates was further decreased. For most isolates, growth was significantly reduced relative to growth at 3 µg/ml. Qualitative observation by light microscopy showed that, in general, colonies on media at all concentrations of genistein were more dense than those of the controls, due to increased mycelial branching. Hyphae also were swollen and twisted. In the case of isolate WR4 only, the mycelium was thin with scant growth at 10 µg/ml.

Influence of genistein on production of zoosporangium. Zoosporangium production was significantly suppressed by genistein in all isolates and, for some isolates, by concentrations of genistein as low as 0.01 µg/ml (Fig. 1). Isolate WR4 was the most sensitive, with zoosporangium production suppressed by approximately 50% at a concentration of 0.01 µg/ml, whereas less sensitive isolates were notably suppressed at 0.1 µg/ml or higher. Regressions across concentrations for individual isolates were: WR4, $F = 4.68$, $P \leq 0.05$; and MSU 03-05, $F = 20.31$, $P \leq 0.01$.

Influence of genistein on zoospore release. Zoospore release was strongly suppressed by the presence of genistein in water, and increasing genistein concentration increased the effects observed. At the lowest concentrations (0.01 to 1 µg/ml), isolates differed in their responses to genistein (Fig. 2). Although there was statistically significant variation between isolates at the highest concentration used, very few zoospores were produced by any

TABLE 1. Influence of genistein on the radial growth of *Phytophthora sojae* in solid culture

Isolate	Radial growth of control (mm)	Radial growth under genistein treatment (% control)[x,y]		P[z]
		3 µg/ml	10 µg/ml	
R25	12.0	108.3 a	83.3 a	**
MSU 01-P	14.0	71.4 b	66.7 b	n.s.
MSU 01-976	13.7	68.0 b	48.0 c	**
MSU 02-1365	16.7	88.9 c	61.1 b,c	**
MSU 03-05	12.0	85.4 c,d	58.5 b,c	**
WR4	2.0	100.0 a,d	50.0 c	**

[x] Growth on V8 agar measured after 5 days.

[y] Isolates followed by different letters within a given concentration are significantly different according to Tukey's test at $P \leq 0.05$.

[z] Differences between 3 and 10 µg/ml concentrations for each isolate are significant at $P \leq 0.01$ (**) or not significant (n.s.).

isolate. The intraspecific variation in response to genistein, therefore, was meaningful only at concentrations of genistein at 1 µg/ml and lower. Control values used in percent control calculations for each isolate are followed by significance parameters for regression across concentrations: WR4 146, $F = 12.05$, $P \leq 0.01$; MSU 03-05 1044, $F = 48.85$, $P \leq 0.01$; MSU 02-1365 816, $F = 80.07$, $P \leq 0.01$; and MSU 02-1365-R 554, $F = 88.73$, $P \leq 0.01$.

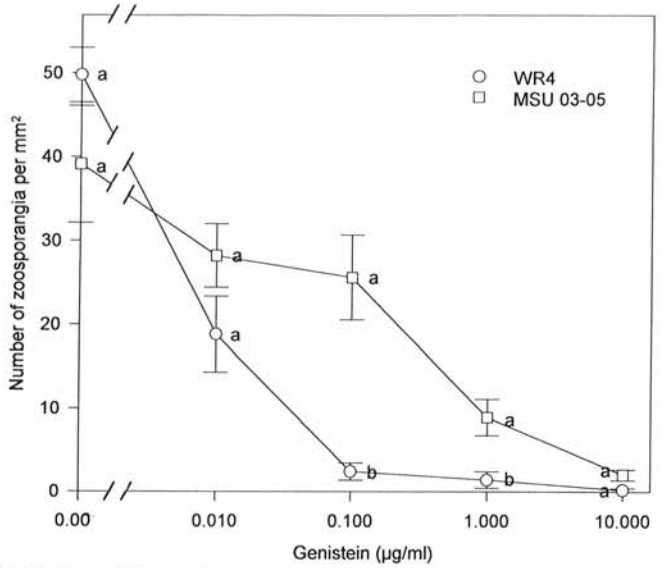

Fig. 1. *Phytophthora sojae* zoosporangium production at different concentrations of genistein. Bars indicate standard error. Within a concentration, points with different letters are significantly different according to Tukey's test at $P \leq 0.05$.

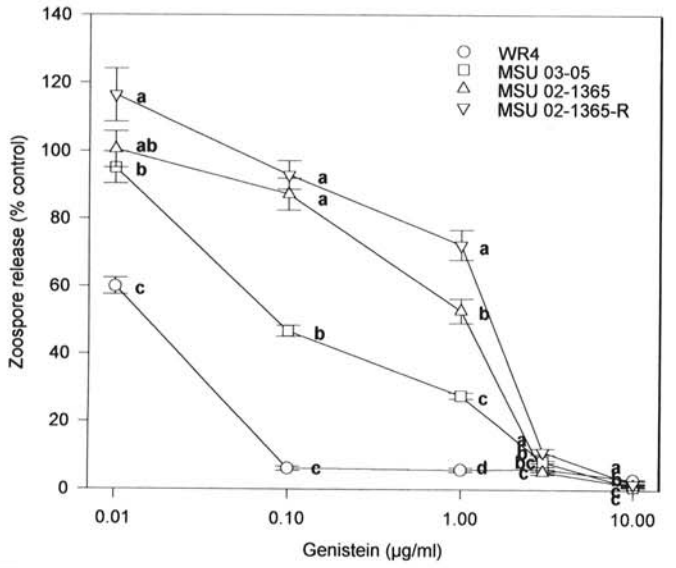

Fig. 2. *Phytophthora sojae* zoospore release at different concentrations of genistein. Data are expressed as percentages of the untreated controls. Bars indicate standard error. Within a concentration, points with different letters are significantly different according to Tukey's test at $P \leq 0.05$.

TABLE 2. Direct germination of zoosporangium of *Phytophthora sojae* produced at different concentrations of genistein

Isolate	Concentration of genistein (µg/ml)[z]					
	0.0	0.01	0.1	1.0	3.0	10.0
MSU 03-05	–	–	+	++	+	–
WR4	–	++	+	+	–	–

[z] – = no direct germination; + = less than 25% germination; ++ = maximal germination, 30 to 50%.

Whereas the presence of genistein in water reduced the release of zoospores, direct germination of zoosporangia was stimulated (Table 2). This stimulation depended on genistein concentration, with isolate WR4 the most sensitive. In most of the zoosporangia that germinated directly, there was no zoospore differentiation of the cytoplasm.

Influence of genistein during zoospore germination and on the overall process of asexual reproduction. When treated with genistein in V8-juice agar during zoospore germination (Fig. 3), the isolates differed widely from each other. Colony formation of isolate WR4 was somewhat inhibited by concentrations as low as 0.01 µg/ml. The response of isolate R25 at 0.1 µg of genistein per ml was not significantly different from its control (*t* test). At 10 µg/ml, the response of both isolates was similar, reaching 50% of their respective controls. Increasing genistein concentration increased the effects observed. Control values (in number of colonies) used in percent control calculations for each isolate are followed by significance parameters for regression across concentrations: WR4 97, $F = 4.52$, $P \leq 0.05$; and R25 146, $F = 63.76$, $P \leq 0.01$.

Exposure to different genistein concentrations during mycelial growth and subsequent zoosporangium and zoospore production caused significant reductions in reproduction (Table 3). The values for the six isolates examined demonstrate the intraspecific

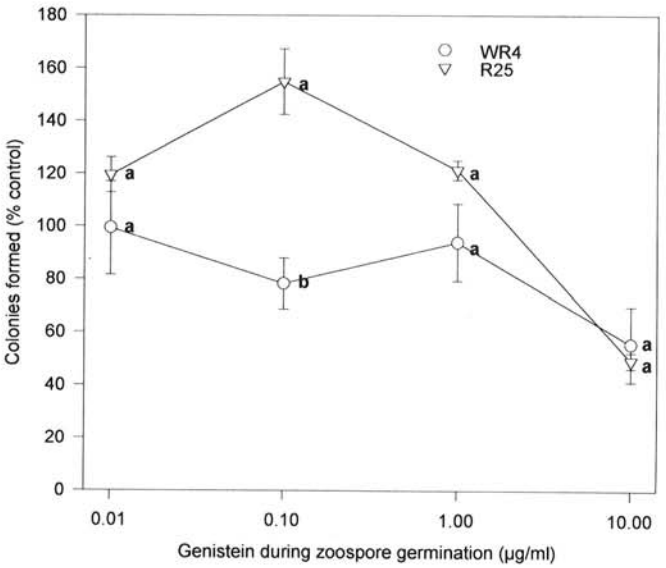

Fig. 3. Number of colonies formed on a solid medium in response to the presence of genistein during germination of *Phytophthora sojae* zoospores. Data are expressed as percentages of the untreated controls. Bars indicate standard error. Within a concentration, points with different letters are significantly different according to Tukey's test at $P \leq 0.05$.

TABLE 3. Formation of colonies in response to exposure to genistein during *Phytophthora sojae* mycelial growth and zoosporangium/zoospore production

Isolate	Control (no. of colonies)	Mycelium: 3^{x-z}		Mycelium: 10^{x-z}	
		Zsp: 0	Zsp: 3	Zsp: 0	Zsp: 10
R4	123	50.7 a	0.8 b	23.6 c	0.5 d
R25	163	55.6 a	1.4 b	38.2 c	1.0 d
MSU 01-P	114	105.8 a	3.5 b	63.4 c	0.0 d
MSU 01	147	108.8 a	4.1 b	73.7 c	0.5 d
MSU 02-1365	90	134.1 a	3.3 b	93.3 c	0.4 d
MSU 03-05	53	120.8 a	10.1 b	28.3 b	0.6 b
Mean	115	96.0 a	3.9 b	53.4 c	0.5 d

[x] Mycelium = genistein concentration in the mycelial growth medium; Zsp = genistein concentration in the zoosporangium/zoospore production medium. Concentrations are in micrograms per milliliter.
[y] Percentage of control.
[z] Data in a given row with different letters are different according to Tukey's test at $P \leq 0.05$.

variation observed. The presence of genistein at 3 µg/ml during mycelial growth resulted in colony formation, which averaged across isolates was only slightly less than the control. In contrast, mycelial growth in the liquid medium containing 10 µg of genistein per ml yielded an average of 53% of the number of colonies of the control. For all isolates examined, the constant presence of 3 µg of genistein per ml during growth and zoosporangium and zoospore production caused a marked decrease (≈90%) in the number of colonies formed, and still fewer colonies were formed in the constant presence of 10 µg/ml.

When genistein was applied during zoosporangium production (Fig. 4), the isolates tested varied in their sensitivity to the lowest concentrations of genistein. Across isolates, however, increasing genistein concentration reduced colony formation. For isolate WR4, the dramatic reduction in colony formation at genistein concentrations as low as 0.01 µg/ml caused the regression analysis across concentrations to be less meaningful and, therefore, less consistent than for the other isolates. Control values (in number of colonies) used in percent control calculations for each isolate are followed by significance parameters for regression across concentrations: WR4 151, $F = 1.44$, $P \leq 0.26$; R25 128, $F = 12.92$, $P \leq 0.01$; MSU 03-05 442, $F = 4.24$, $P \leq 0.07$; and MSU 02-1365 305, $F = 10.53$, $P \leq 0.01$.

Isolates R25 and WR4 were chosen to demonstrate intraspecific variation in colony formation due to the presence of genistein during the entire process of asexual reproduction (excluding mycelial growth) (Fig. 5). Increasing genistein concentration in the media reduced colony formation and typifies the intraspecific variation.

DISCUSSION

This is, we believe, the first report of the influence of the isoflavone genistein on the asexual reproduction of *P. sojae*. Our study showed that, in general, cultures of *P. sojae* exposed to genistein concentrations of 0.01 to 10 µg/ml exhibited decreased mycelial growth, zoosporangium production, release of zoospores, and colony formation. Regardless of reproduction stage tested, except mycelial growth, intraspecific variation in response to genistein was expressed most dramatically at 0.01 to 1 µg of genistein per ml. It should be noted that colony formation is a reflection of zoospore germination, rather than a quantitative equivalent, because a given colony may actually encompass more than one germinating zoospore.

Rivera-Vargas et al. (16) demonstrated strong inhibition of growth of *P. sojae* at 60 to 120 µM concentrations of genistein and a completely fungicidal effect at 240 µM. These results prompted Rivera-Vargas et al. to suggest that genistein may play a role complementary to the antibiotic role of glyceollin in *P. sojae* hypersensitive lesions (9,16). The hypothesis was supported by the demonstration that comparable concentrations of genistein are present in all infected soybean seedling tissues (11). In our study, inhibition of growth of all isolates occurred at the highest concentration tested, 10 µg/ml (37 µM). At low concentrations of genistein, we saw the same subtle effects on morphology described by Rivera-Vargas et al. (16), such as increased branching, swelling, and twisting of hyphae. These changes also occurred at 10 µg/ml despite a reduction in hyphal extension.

Genistein, like other chemoattractants (5,6,13), stimulates rapid, synchronized encystment before germination. However, genistein, unlike other chemoattractants, causes effects in vitro at nanomolar, rather than micromolar, concentrations. Mechanisms for influencing zoospore germination by exposure to genistein are unknown, but there are indications that isoflavones are capable of stimulating spore germination and hyphal growth of arbuscular mycorrhizal fungi in vitro (8,14,18).

The reproductive behavior of *P. sojae* is strongly affected by prehistory conditions experienced during mycelial growth. When

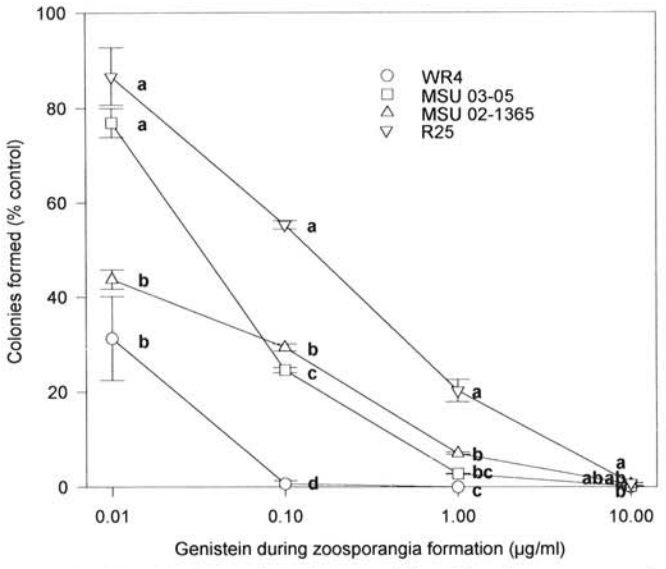

Fig. 4. Number of colonies formed on a solid medium in response to the presence of genistein during induction of *Phytophthora sojae* sporulation. Data are expressed as percentages of the untreated controls. Bars indicate standard error. Within a concentration, points with different letters are significantly different according to Tukey's test at $P \leq 0.05$.

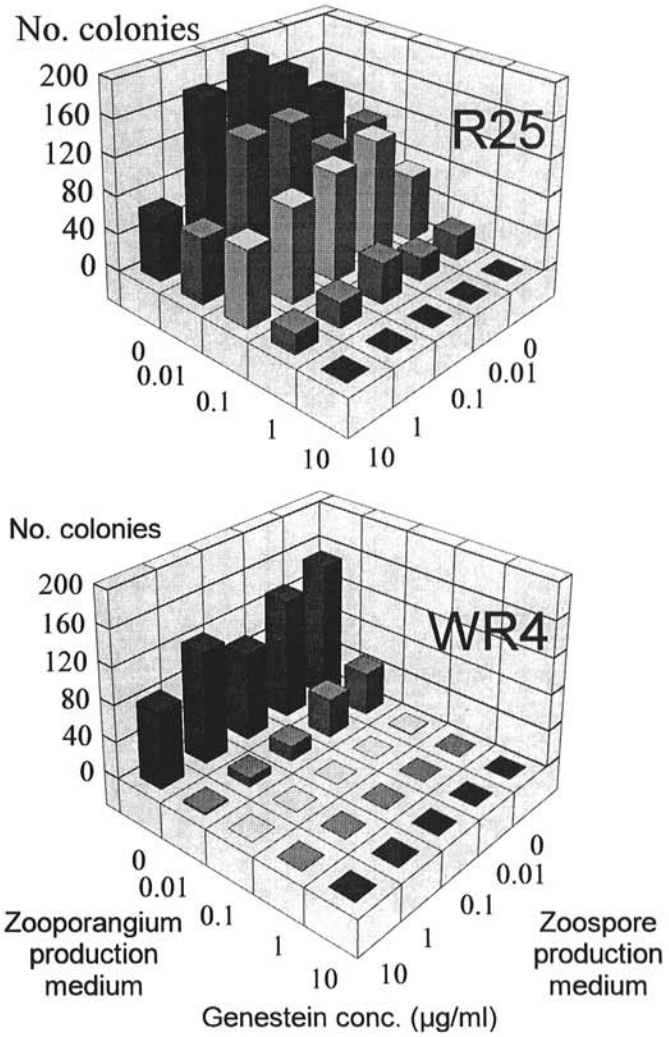

Fig. 5. Responses of two *Phytophthora sojae* isolates to genistein applied variously during the entire process of asexual sporulation. All means were significantly separated according to Tukey's test at $P \leq 0.01$.

mycelium was grown in the presence of genistein, which was subsequently removed during zoosporangium and zoospore production, the number of colonies formed at 3 μg/ml was reduced only slightly, while mycelium grown at 10 μg/ml resulted in markedly reduced colony formation. When genistein was present throughout mycelial growth and subsequent zoosporangium/zoospore production, a dramatic reduction in colony formation was seen at 3 μg/ml, and an almost complete suppression occurred at 10 μg/ml across all isolates examined. Strong prehistory effects also have been shown for zoospore germination of *Pythiaceae* (15).

Genistein had a definitive negative influence on asexual reproduction, which may be related to the ecological behavior of the pathogen. The decreases in zoosporangium formation and release of zoospores resulted in a reduced number of colonies formed even at the extremely low concentration of 0.01 μg/ml. Depending on the timing of exposure to genistein at 10 μg/ml, asexual reproduction was markedly reduced or almost completely prevented. These reductions suggest that genistein might be involved in the regulation of asexual sporulation and, consequently, may influence *P. sojae* population levels. *P. sojae* appears capable of adapting to genistein exposure. At concentrations inhibitory to zoospore release, direct germination of zoosporangia occurred, reaching its maximum at 1 μg/ml for isolate MSU 03-5 and at 0.01 μg/ml for isolate WR4.

P. sojae exhibits strong intraspecific variation in response to genistein, even at concentrations as low as 0.01 to 1 μg/ml. The possible adaptive significance of this variation is unclear. Aggressive isolates of *P. sojae* may have developed a fitness trait to concentrations of genistein that limit development of unadapted individuals. Adaptation to genistein may be associated with virulence as demonstrated by the opposing responses of WR4, an avirulent isolate, and R25, a highly aggressive isolate. Given the intraspecific variation observed for all processes tested, it is probable that other isolates would demonstrate responses as different as the two isolates used in this experiment.

Although mechanisms controlling responses to genistein have not been determined, the dramatic responses we obtained in vitro suggest that this compound may well exert an influence on the development of *P. sojae* in nature.

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